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LEUPHASYL®

CODE: PD080

Date: October 2012 Revision: 12

TARGET ACHIEVED: SMOOTH AND YOUNG SKIN FOR LONGER

A NEW SYNTHETIC COSMETIC INGREDIENT

SUMMARY

Lipotec has developed a new peptide to reduce expression wrinkles and that offers two advantages:

- A new and alternative *in vitro* mechanism to fight expression wrinkles
- Additive / Synergistic effect to complement the action of ARGIRELINE®

LEUPHASYL®'s mechanism mimics *in vitro* the natural mechanism of enkephalins: it couples to the enkephalin receptor, on the outside of the nerve cells. When LEUPHASYL® couples to the receptor, a conformational change initiates a cascade inside the neuron that results in a decrease of its excitability: the nerve cell's activity is "turned down" and the release of acetylcholine is modulated. Muscle contraction will be relaxed and therefore, expression wrinkles will be diminished.

GENERAL DESCRIPTION

One of the most striking signs of skin aging is increased wrinkling of the face. This can occur naturally over time and is identified by certain biochemical, histological and physiological changes that are enhanced by environmental exposure. There are other secondary factors that can cause characteristic folds, furrows and creases of the face. These include the constant pull of gravity, frequent and constant positional pressure of the skin of the face (e.g. during sleep) or repeated facial movements caused by the contraction of the muscles of facial expression.

Muscles are contracted when they receive acetylcholine released from inside a vesicle in a process known as neuronal exocytosis.

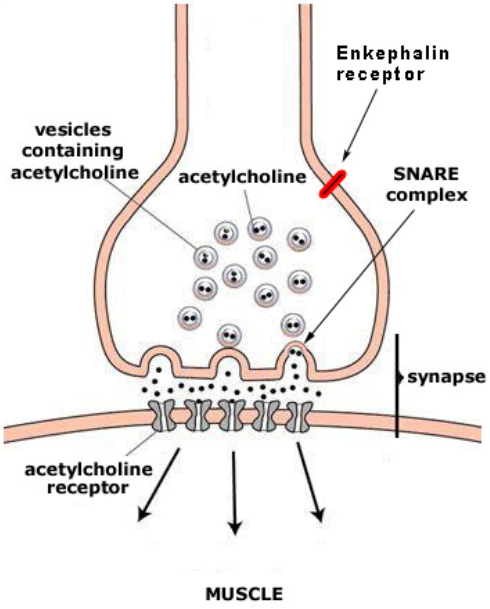


Fig. 1. Neuronal exocytosis

LEUPHASYL® is an enkephalin modified for enhanced stability that provides a modulation of acetylcholine release from neuron cell cultures. This may attenuate muscle contraction in vivo, preventing the formation of lines and wrinkles.

In order to have an effective muscle contraction, two events are required:

a) Vesicles must be loaded with Acetylcholine
b) Calcium ions must enter the neuron to trigger vesicle fusion

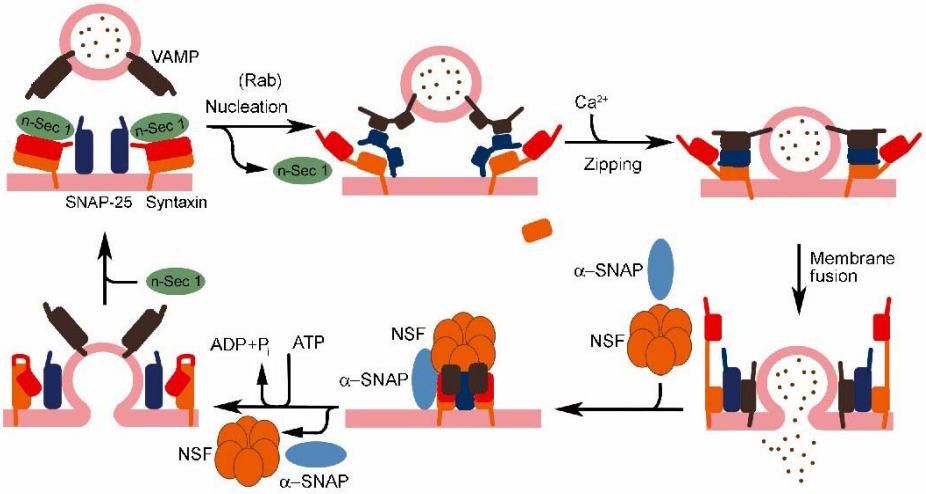


Fig. 2. Vesicle fusion is Ca^{2+} dependent

Natural enkephalins are endogenous opioids: they inhibit neuronal activity. Their receptors are on the outside of neurons, coupled to G proteins of the inhibiting type (Gi). Enkephalin molecules docking in these receptors results in the release of the G protein subunits (alpha, beta, gamma). These subunits close Ca^{2+} channels and open K^{+} channels. Preventing the entry of Ca^{2+} into the neuron avoids vesicle fusion and consequently inhibits acetylcholine release across the synapse to the muscle.

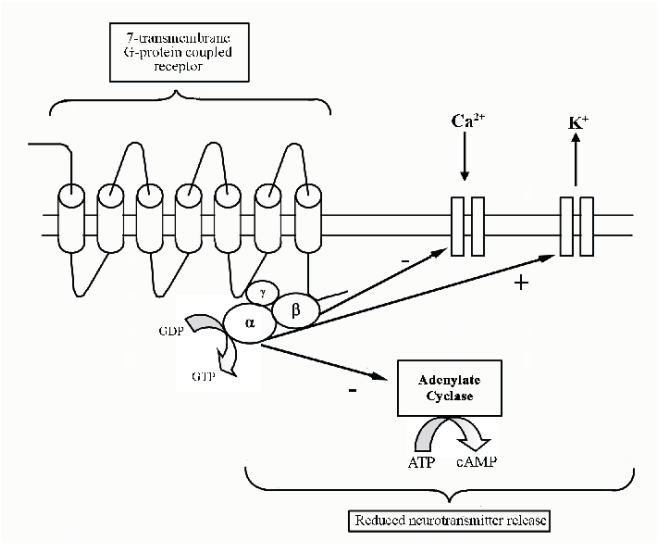


Fig. 3. Enkephalin docking closes Ca^{2+} channels

From the electrical point of view, the neuron is at its resting potential at about -60 mV (see figure 4). It is surrounded by a high Na^+ and low K^+ concentration environment. How does the neuron send its signals?

Point 1: the neuron "activates" when it receives a signal that opens the Na^+ channels. Positive charge rushes into the neuron, making the inside more and more positive. This causes membrane potential to rise towards a positive voltage.

Point 2: Na^+ channels are "voltage activated" which means that when the threshold is reached (approx –40 mV) the channels open completely, enabling a massive entry of Na+. Membrane potential rises fast.

Point 3: Na^+ channels also have an inactivation mechanism: once the Na^+ entry has been maintained for a certain time (+40 mV is reached), the channel closes and inactivates. In order to return to its resting potential, the neuron needs to remove positive ions from the inside. This is accomplished by opening K^+ channels. Since K^+ concentration outside is smaller, K^+ ions rush outside, taking the excess positive charge out of the neuron. The membrane potential decreases.

This localized change in membrane potential is called an *action potential*, and it is transmitted along the neuron, all the way down to the neuron terminal. At the neuron terminal we find Ca^{2+} channels instead of the Na^+ channels so the wave of action potential will trigger the entry of Ca^{2+} into the neuron, which is what we need for vesicle fusion.

Enkephalins keep the Na+/Ca2+ out and enable K+ to leak out, maintaining the neuron in its resting state.

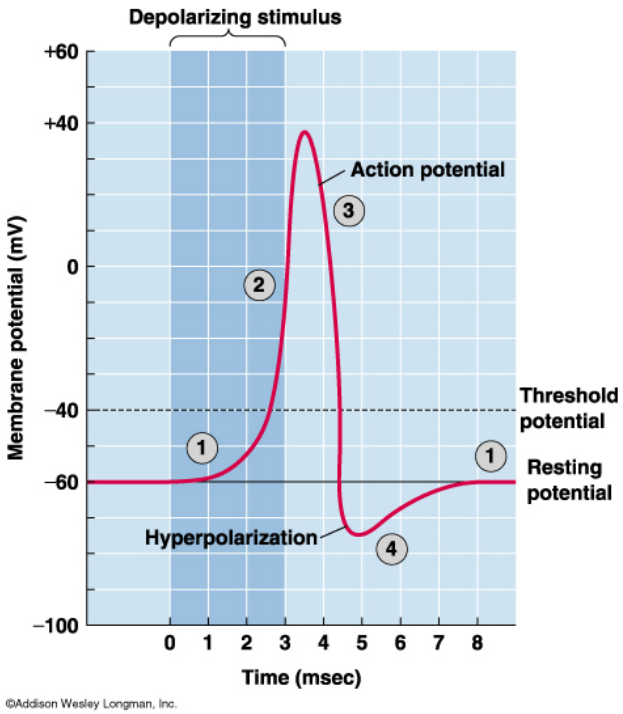


Fig. 4. An action potential

PROPERTIES AND APPLICATIONS

- LEUPHASYL® reduces the depth of wrinkles on the face caused by the contraction of muscles of facial expression, especially in the forehead and around the eyes.

- LEUPHASYL® targets *in vitro* the wrinkle-formation mechanism of expression wrinkles in a new way, offering an alternative to peptides like ARGIRELINE®.

LEUPHASYL® can be incorporated in cosmetic formulations such as emulsions, gels, sera, etc., where removal of the deep lines or wrinkles in the forehead or around the eyes area is desired.

TECHNICAL INFORMATION

PRODUCT SPECIFICATIONS

LEUPHASYL® Solution	
Code:	PD080
INCI name:	Water, Glycerin, Pentapeptide-18
Appearance:	Translucent solution
Contents:	0.05% LEUPHASYL® powder
Preservative:	0.5% Caprylyl Glycol

PROCESSING AND DOSAGE

LEUPHASYL® is presented as **LEUPHASYL® Solution**, an aqueous solution containing 0.5 g/L of the peptide powder. It can be incorporated at the final stage of the manufacturing product, provided the temperature is below 40 °C. Taking into consideration the concentration of peptide in **LEUPHASYL® Solution**, it is recommended that 3% to 10% of the solution is present in the final formulation in order to obtain significant anti-wrinkle activity.

STORAGE AND SHELF LIFE

LEUPHASYL® Solution must be kept in a cool, dark and clean place to ensure a shelf life of at least 24 months.

LEUPHASYL® Solution is best kept in the refrigerator. In rare cases, refrigerated storage of **LEUPHASYL® Solution** can cause precipitation of the preservative. This does not affect the integrity of the product.

SAFETY

The toxicological profile of LEUPHASYL® for cosmetic purposes was assessed only "in vitro" or on a panel of human volunteers (in Spain). A full toxicological report and a summary of all the safety tests performed are available on request.

All tests were performed using solutions of LEUPHASYL® at the desired concentrations.

In vitro tests

Citotoxicity test on human dermal fibroblasts
No signs of citotoxicity were observed.

Citotoxicity test on human epidermal keratinocytes
The results showed no signs of citotoxicity at the concentrations assayed.

Genotoxicity test (Ames test)
The results showed no genotoxicity under the conditions assayed.

Ocular Irritation (HET-CAM test)
The product is potentially not irritating for the eyes.

In vivo tests

Acute oral toxicity test
Analysis design allowed to conclude that LD_{50}>2500 mg/Kg body weight in rats and therefore LEUPHASYL® shows no acute oral toxicity at the dosage tested. This test is compulsory for any new chemical entity according to the Dangerous Substances Directive 67/548/EC.

Skin irritation (Patch Test)
The test was performed on 10 human volunteers, aged 18 to 70, both sexes, phototype Fitzpatrick I to V. LEUPHASYL® Solution (20 µl) was applied on the back, under an occlusive patch for 48 hours +/- 5 hours. Skin examination was performed visually under standard "daylight" source, 15 minutes (or more if some redness appeared after patch removal), then 24 hours +/- 2 hours after patch removal.
The results of the individual daily irritation score (Idis) and the mean daily irritation score (Mdis) were 0, and the conclusion was that LEUPHASYL® Solution has a very good skin compatibility

EFFICACY DATA

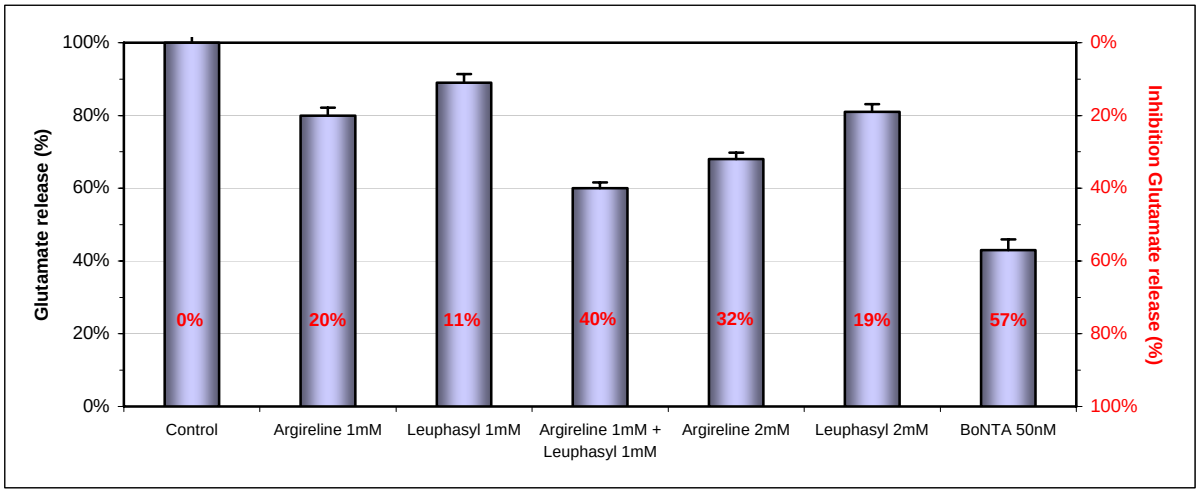
In vitro tests

Modulation of glutamate release in a neuron cell culture

Inhibition of glutamate release by depolarized neuron cells is a validated cell assay for measuring the potential activity of compounds on the inhibition of neuronal exocytosis. The K^+-induced depolarization of hippocampal cultures in the presence of extracellular Ca^{2+} results in the release of glutamate, which is the most abundant excitatory neurotransmitter in the nervous system.

A primary cell culture of neurons was incubated with tritiated glutamine during 3 h in order to load them with radiolabelled glutamate. Afterwards, the excess of glutamine was rinsed off, and they were incubated with the test items for 1 h at 37 ºC. The release of radiolabelled glutamate is made by depolarization with buffered 75mM KCl/2mM $CaCl_2$ in physiologic buffer for 10 min at 37 ºC. The culture media was collected and the quantity of radiolabelled glutamate was quantified using a scintillation counter. Values obtained are the average of 6 determinations. Untreated neuron cultures were used as a negative control and cultures treated with Botulinum Toxin A (BoNT A) were used as positive controls.

The release of glutamate from the neurons is measured in order to compare the *in vitro* activities of the anti expression-wrinkle peptides ARGIRELINE® and LEUPHASYL®.



The combination of LEUPHASYL® with ARGIRELINE® showed a higher inhibitory potential of glutamate release than the inhibitory potential resulted from the addition of each single product, which means that both peptides show a synergistic effect *in vitro*, their mechanisms are independent and their effects can be added.

Note: A previous preliminary test of modulation of glutamate release in a neuron cell culture was performed with fewer samples (*RT-PD080, January 2006, Rev. 4*). The present test is definitive and has been carried out with a higher number of determinations (n=6).

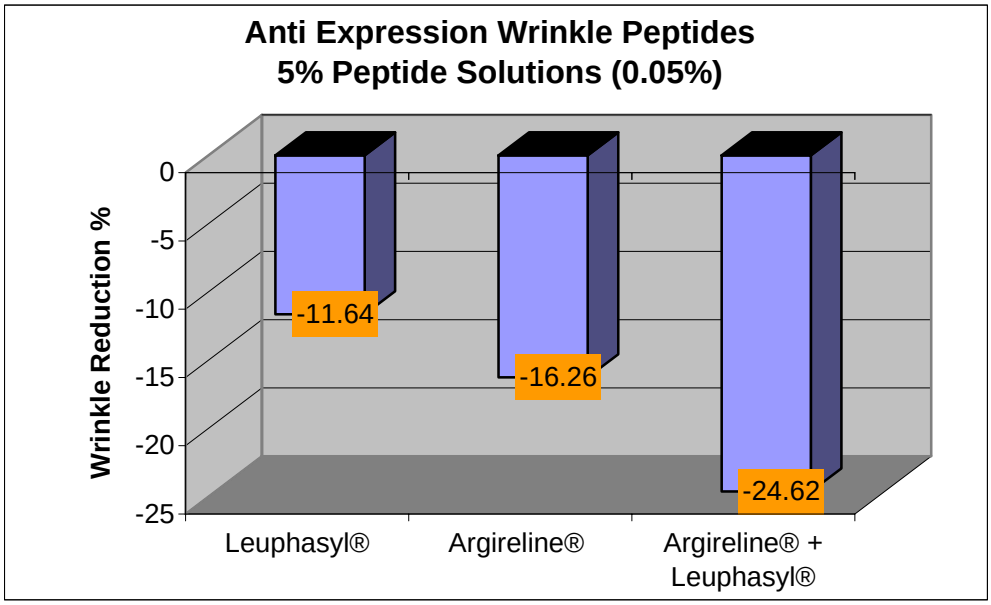
In vivo test

In order to prove an alternative mechanism to Lipotec's other expression anti-wrinkle, ARGIRELINE®, parallel tests were performed in order to compare both peptides. Tests were performed using a placebo cream.

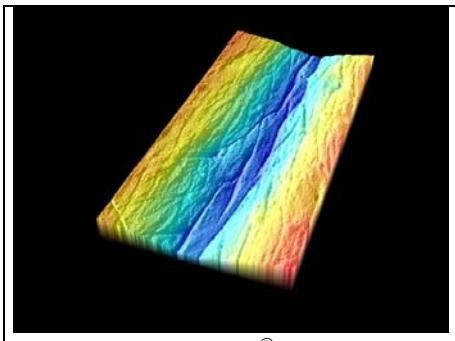
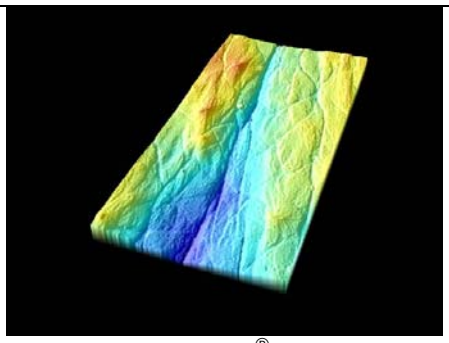
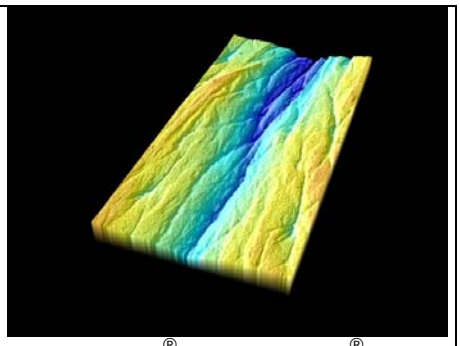
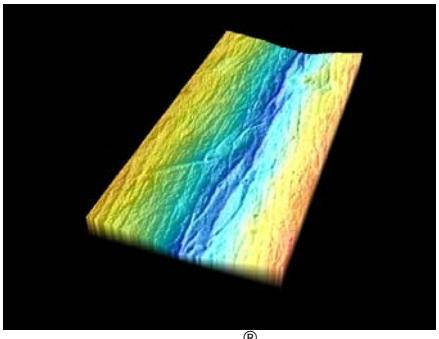
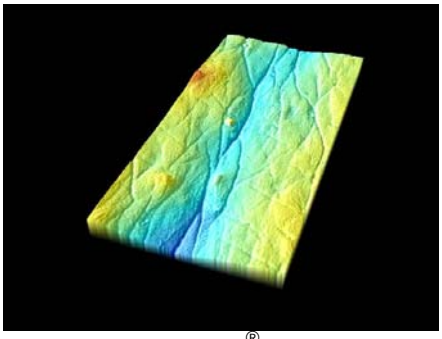
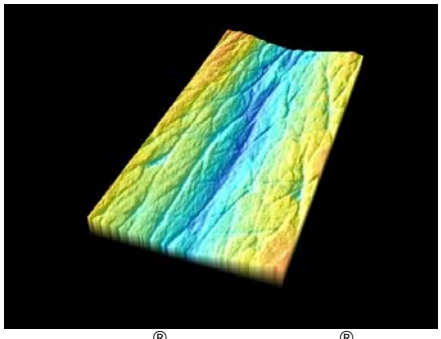
Efficacy of LEUPHASYL® as an anti-wrinkle agent was evaluated by taking silicon imprints of the wrinkles around the eyes of healthy volunteers. These wrinkles are expression wrinkles. A cream containing 5% LEUPHASYL® Solution (0.05%) was applied twice daily around the eyes of 14 volunteers, aged 39 to 64, for 28 days. A **decrease of 11.64%** was accomplished, with maximum values up to –23.55%.

A cream containing 5% ARGIRELINE® Solution (0.05%) was applied twice daily around the eyes of 14 volunteers, aged 39 to 63, for 28 days. A **decrease of 16.26%** was accomplished, with maximum values up to –31.80%.

A third test was performed using a combination of both actives in order to evaluate a possible synergy or additive effect. A cream containing 5% ARGIRELINE® Solution (0.05%) + 5% LEUPHASYL® Solution (0.05%) was applied twice daily around the eyes of 15 volunteers, aged 39 to 63, for 28 days. A **decrease of 24.62%** was accomplished, with maximum values up to –46.53%. Both peptides show additive effects due to their complementary mechanisms.



The following are representative images of the silicon replicas:

		
LEUPHASYL® Day 0	ARGIRELINE® Day 0	LEUPHASYL® + ARGIRELINE® Day 0
		
LEUPHASYL® Day 28	ARGIRELINE® Day 28	LEUPHASYL® + ARGIRELINE® Day 28

Dark blue colour indicates maximum depth – bright red colour indicates max height

GENERAL PRODUCT INFORMATION

Trade name	LEUPHASYL® SOLUTION
Product code	PD080

INGREDIENTS

INCI name	CAS No	EINECS No
WATER (AQUA)	7732-18-5	231-791-2
GLYCERIN	56-81-5	200-289-5
PENTAPEPTIDE-18	64963-01-5	-
CAPRYLYL GLYCOL	1117-86-8	214-254-7

Note: Graphs and photographs are available for customer use provided that the final product contains the same concentration of active as the formulations in our test. Customers must request written permission for use of the graphic material and/or ingredient tradenames to Lipotec. Customers are responsible for compliance with local and international advertising regulations.
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